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Genome Organization and Transcription Response to Harvest of Two Metallothionein-Like Genes in *Agaricus bisporus* Fruiting Bodies

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Metallothioneins are a class of small cysteine-rich proteins that have been associated with increased tolerance to metal and oxidative stresses in animals, plants, and fungi. We investigated a metallothionein-like (*mt*-like) gene shown previously to be upregulated in fruiting bodies of the fungus *Agaricus bisporus* in response to post-harvest storage. Analysis of an *A. bisporus* genomic DNA cosmid library identified two similar *mt*-like genes (*met*1 and *met*2) arranged as a bidirectional gene pair transcribed from the same promoter region. The promoter contained regulatory elements including 9 metal responsive elements and a CAAT box region 220 bp downstream of *met*1 that showed striking similarity to a feature in *Coprinopsis cinerea mt*-like gene promoters. Transcriptional analysis showed that both *met* genes are significantly and rapidly (within 3 hours) upregulated during post-harvest storage and expression is significantly greater in stipe and cap tissues compared with the gills. However, a strong directionality of the promoter was demonstrated, as transcript levels of *met*1 were at least two orders of magnitude greater than those of *met*2 in all samples tested.

Keywords: Metallothionein, *Agaricus bisporus*, bidirectional promoter

Regulation of cytosolic metal concentrations is essential for the maintenance of cellular activity in prokaryotes and eukaryotes. Metal ions, such as iron, copper, and zinc, are vital cofactors for a wide range of proteins and enzymes where the metal moiety can function in ligand binding and reductive and oxidative reactions [3]. An insufficient supply of metals to the cell can cause disruption to cellular functioning and reduced fitness of the whole organism [33]. However, an excess of metal ions can inhibit growth and cause damage to membranes, proteins, and nucleic acids through the creation of reactive oxygen species [26]. The ability to regulate the concentration and availability of metal ions in the cytosol is important during the normal dynamic turnover experienced by the cell [3, 32]. This ability is particularly important when the cell undergoes rapid physiological change that may alter the level of cytosolic metal ions, for example, during cell differentiation, in response to damage stress or due to the influx of heavy metals into an environment [7, 29].

Organisms have evolved to transport and chelate metal ions in order to deliver them to where they are needed or to sequester them to prevent cellular damage [10]. Glutathione-induced proteins (phytochelatins) and metallothioneins have been widely implicated in the intracellular binding of copper, zinc, and cadmium ions in fungi, plants, and animals [7, 10, 30]. Both are small cysteine-rich proteins in which metal ions interact with the thiol group of the cysteine amino acids. Whereas metallothioneins are encoded by genes, phytochelatins are produced by proteins from the polymerization of γ-glutamyl-cysteine subunits [9, 30]. Metallothioneins have been well studied in yeast systems, where their regulation has been linked with resistance to heavy metals and oxidative stress [6, 28, 26, 49]. Studies have identified metallothioneins from filamentous fungi, in particular with regard to mycorrhizal associations and the potential for increased metal tolerance provided by the fungus in contaminated soils [1, 2, 24, 30].

The accumulation of metals in fruiting bodies of *Agaricus* spp. is well documented [42], and the UK Food Standards Agency has expressed concern for the concentration of cadmium in *Agaricus arvensis* mushrooms in the UK (http://www.

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foodstandards.gov.uk/multimedia/pdfs/envirocontam0703). Previous work on *Agaricus bisporus* has identified two metallothionein-like cysteine-rich peptides [12, 31]. The *A. bisporus* metallothionein MT-AGABI, Accession No. P04358, was shown to be similar to the mammalian class of metallothioneins and was induced by the presence of copper in the medium, but not by zinc or cadmium [31]. This was supported by the partial purification of cadmium-binding molecules from *A. bisporus* fruiting bodies that did not include a metallothionein [14]. A second metallothionein-like cDNA, *met*1 Accession No. AJ312127, was shown to be upregulated in *A. bisporus* fruiting bodies that had been harvested and stored for 2 days [12]. Metal binding ability was not demonstrated, but the gene shows similarity with other fungal metallothioneins [2, 24]. Harvested mushrooms are exposed to a range of stresses that have been shown to induce metallothionein expression in other organisms, including damage and oxidative stress [6, 26]. Harvested fruiting bodies continue to develop and release spores despite being isolated nutritionally from the mycelium, causing loss of product quality. Although, *met*1 is upregulated following harvest, the role of the gene in the biology of the post-harvest mushroom is unknown. The research in this paper aims to further characterize the *A. bisporus met*1 gene by obtaining genomic DNA sequence information of the gene and promoter region, and to assess the expression of the gene in greater detail during fruiting body formation and post-harvest storage in order to better understand its regulation and role in the mushroom.

Materials and Methods

Strains and Culture Conditions

Agaricus bisporus strain A15 (Sylvan, UK) was used throughout the study. *A. bisporus* cultures were grown on composted wheat straw according to commercial practice at the Warwick HRI BioConversion Unit.

Fruiting bodies were sampled at morphogenetic stage 2 [18]. Further harvested mushroom samples were stored in a controlled environment, 18°C and 95–95% relative humidity, for three time courses. Mushrooms were stored over (i) 0 to 24 h time course post-harvest (three hourly intervals), (ii) 0 to 5 day time course following harvest (24 hourly intervals), and (iii) 0 to 48 h post-harvest (24 h intervals), with mushrooms dissected into stipe, cap, and gill tissues. Three replicate mushrooms were taken for each sampling point, frozen under liquid nitrogen, and stored at −80°C.

Cloning of the *A. bisporus met*1 gene

The *met*1 gene was isolated from an *A. bisporus* C54-carb8 genomic Lawrist cosmid library [49]. Cosmid library primary screening was performed on 87 pooled batches comprising DNA from 96 *E. coli* clones containing Lawrist cosmids by PCR with specific MET primers designed from the *met*1 cDNA sequence [12]. PCR products of the correct size were sequenced and verified against the *met*1 cDNA sequence. A secondary colony hybridization screen was performed with the 96 *E. coli* clones from the positive pooled batch identified in the primary screen and the cosmid clone containing the *met*1 sequence was isolated according to the Qiagen Tip20 plasmid purification protocol (Qiagen, Germany). *met*1 cDNA was isolated from a phagemid clone by treatment with *Hin*dIII and *Bam*HI, separated by agarose gel electrophoresis, and purified following the Qiagen gel purification column protocol. [α-^{32}P]dCTP randomly labeled *met*1 cDNA (*Redi*prime kit; Amersham Pharmacia Biotech., Buckinghamshire, UK) was used as a probe and colony blot hybridizations were carried out according to established methods [39]. The *met*1 gene sequence and upstream and downstream flanking regions were obtained from the purified cosmid DNA by the primer walking method. Sequencing reactions were conducted using the Warwick HRI Genome Centre sequencing service at the University of Warwick.

Sequence Analysis

Analysis of cosmid sequences was carried out using tools available in the DNAStar software suite (Lasergene). Web-based homology (Blast) and pattern (Prints, Blocks) searches were carried out using tools accessed through the EBI (http://www.ebi.ac.uk/index.html) or ExPaSy (http://www.expasy.ch/tools/) Web sites. Basidiomycete-specific genome databases available at the Broad Institute (http://www.broad.mit.edu/) and JGI (http://www.jgi.doe.gov/) were used to identify sequences that shared similarity with *A. bisporus met* sequences in other fungi. ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/) was used to align sequences and the Transfac database (http://www.gene-regulation.com/pub/databases.html) was used to search promoter regions for possible regulatory elements.

RNA Isolation

RNA was isolated from mushroom tissues according to established phenol/chloroform extraction protocols [39]. Absorbance measurements at 260 nm and 280 nm were used to assess RNA concentration and purity. RNA integrity was determined with formaldehyde agarose gel electrophoresis [38]. For experiments involving reverse transcriptase, RNA samples were treated with RQ1 RNAse-free DNAse enzyme (Promega, Southampton, UK) according to the manufacturer's instructions.

Northern Analysis

Total RNA, ~10 μg, from each sample was separated by formaldehyde agarose gel electrophoresis and immobilized onto nylon membranes carried out using established protocols [39]. Hybridization was carried out using the randomly primed [α-^{32}P]dCTP *met*1 cDNA probe as described previously and post-hybridization washes as per established protocols [39]. The *Agaricus bisporus* 28S rRNA gene was used as a loading control as described previously [11, 13].

Quantitative RT–PCR (qRT–PCR)

The ABI Prism 7900HT sequence detector (TaqMan) and SYBR Green fluorescent reporter dye were used to measure transcript levels. Reverse transcriptase reactions were carried out on three replicate samples for each time point using the Thermoscript RT–PCR system (Invitrogen, Life Technologies, Paisley, UK). Reactions were performed in a 20 μl total volume and consisted of 50 ng/μl random hexamers, 1 μg of total RNA, 4 μl of 5× Thermoscript buffer, 1 μl of RNaseOUT (40 U/μl), 1 μl of 0.1 M DTT, and 1 μl of Thermoscript reverse transcriptase (15 U/μl). Reactions were carried out at 25°C for 10 min,

followed by 50 min at 50°C, and terminated at 85°C for 5 min. All samples were treated with RNAse H according to the manufacturer's instructions and each reaction mixture was diluted to a 100 µl final volume with sterile ultrapure water.

Quantitative PCR reactions (15 µl total volume) were prepared, consisting of 1 mM of each primer, 20 ng of cDNA sample, and 7.5 µl of 2× SYBR Green PCR Mix (Applied Biosystems, Warrington, UK). Three technical replicates were prepared for all sample and standard reactions. PCR cycling conditions consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles 95°C 15 s and 60°C for 1 min, followed by a dissociation step of 95°C for 15 s, 60°C for 15 s, and increase to 95°C with a 2% ramp rate. The dissociation step was included for melting curve analysis to detect whether primer dimers or nonspecific products were present in the reaction. Standard curves were generated with cDNA (0.625 ng/µl to 20 ng/µl) from a whole 2-day stored mushroom, as this had been shown to contain *met*1 transcripts previously [12]. The primer sets used were MET1F 5'-TGCCTGCGCCAACAACT-3', MET1R 5'-GGACATGCCACAGTGTTGGTT-3'; MET2F 5'-GCAATTGTGGA GACGCGTAA-3', MET2R 5'-CGCATCTATGGTCCGTCATT-3'; and 18SrRNAF 5'-ACAACGAGACCTTAACCTGCTAA-3', 18SrRNAR 5'-GACGCTGACAGTCCCTCTAAGAA-3'. Primers were designed using the Primer Express software version 2.0 (Applied Biosystems, Warrington, UK).

The ABI PRISM sequence detector software (SDS, version 2.0) was used to determine the cycle threshold of each sample (Ct-Target), which was normalized to the cycle threshold of the 18S rRNA qRT–PCR product (Ct-Control) for the same sample [13, 16]. The ΔCt equation ($\Delta Ct=2^{(CtControl - CtTarget)}$) was used to calculate the amount of each target transcript relative to the amount of 18S rRNA.

Three control treatments were included: (i) water control: sterilized diethylpyrocarbonate (DEPC)-treated water replaced the cDNA sample in order to detect environmental DNA contamination and primer-based artefacts; (ii) DNAse-treated RNA control: to ensure no contaminating DNA was in the RNA samples; and (iii) reverse transcriptase control: the reverse transcriptase reaction was carried out in the absence of random hexamers, where qPCR was carried out as described, and this control would detect contaminating DNA and the possibility of active reverse transcriptase present during qPCR.

Transcript levels obtained by qRT–PCR were analyzed statistically using analysis of variance (ANOVA) for each experiment (during post-harvest 0–24 h, 0–5 days, and 0–48 h between different tissues) separately. The data were subjected to a logarithm (base 10) transformation prior to analysis to satisfy the ANOVA assumption of homogeneity of variance. An F-test was used to assess the significance of the overall treatment effects, and the significance of differences between individual treatment means was assessed by comparison with appropriate standard errors of differences (SEDs). Treatment differences noted in the text are significant at the 5% level unless stated otherwise.

RESULTS

The *A. bisporus met*1 Gene

The full-length *met*1 gene sequence was determined from the analysis of *A. bisporus* genomic DNA isolated from an *A. bisporus* C54-carb8 genomic Lawrist cosmid library. Exon/intron boundaries were identified by comparison with the cDNA sequence and determination of conserved *gtnng* and *yag* intron boundaries [44].

The *met*1 gene sequence consists of a 165 bp open reading frame with 2 introns, a 52 base 5'-untranslated region (UTR), and a 274 base 3'-UTR (Fig. 1A). The introns and 5'- and 3'-UTRs were confirmed by cDNA sequence analysis. Both introns were 55 bp in length and appeared to have normal splicing sites. A putative polyadenylation signal [20] was found in the 3'-UTR region, 27 bases from the polyadenylation start site (Fig. 1A). *In silico* sequence analysis revealed the presence of a second putative paralogous *met* gene (*met*2, Accession No. AJ312128) located 568 bp upstream from the previously obtained *met*1 cDNA sequence (Fig. 1A). The deduced translated sequence of *met*2 consisted of 59 amino acids (13 cysteines) and was shown to share 57.8% identical amino acid residues with *met*1, which was composed of 54 amino acids with 14 cysteines (Fig. 1B). The position of 13 cysteine amino acids was conserved between the two MET proteins. The genes appeared to be arranged as a bidirectional gene pair divergently transcribed from a bidirectional promoter region [44]. The putative *met*2 gene sequence consists of a 180 bp open reading frame with two introns (59 bp and 50 bp in length). The first intron was located 15 bp from the translational start site as observed with *met*1, but the base composition and length of the introns were not comparable. The second intron of *met*2 did not show any similarity in structure or position with *met*1.

The 568 bp region separating the two *met* genes (Fig. 1A) (Accession No. AJ312127) was analyzed for transcription regulatory motifs. A conserved 11 bp motif containing a putative TATA box (GATATAAAAGG) was identified at positions −102 and −76 from the translation start of *met*1 and *met*2, respectively. A putative CAAT-box was identified at position −220 from the translation start of *met*1, but no similar sequence was found close to *met*2. Furthermore, nine metal responsive element motifs (MRE) were identified matching the MREs described in the *Pleurotus ostreatus* laccase gene promoter [15], which are similar to the recognized MRE consensus sequence TGCRCNC [43], 3 pairs of which occupied overlapping sequences (Fig. 1A).

Similarity searches using the *met*1 sequence (Blastn, BlastX, and BlastP) carried out through the EBI database showed no similarity with any previously identified gene. BlastP with *met*2 showed similarity with numerous metallothionein-like (*mt*-like) genes, including *Gigaspora margarita* (p-value=3.9e-08, Accession No. Q8NJ33 [24]). Both *A. bisporus met* sequences were then used to search the databases of recently sequenced basidiomycete genomes of *Coprinopsis cinerea*, *Laccaria bicolor*, *Phanerochaete chrysosporium*, and *Postia placenta*. Two similar *met* genes were identified in each fungus, with the exception of *P. placenta* where three similar peptide sequences with some similarity were identified. However, it was not

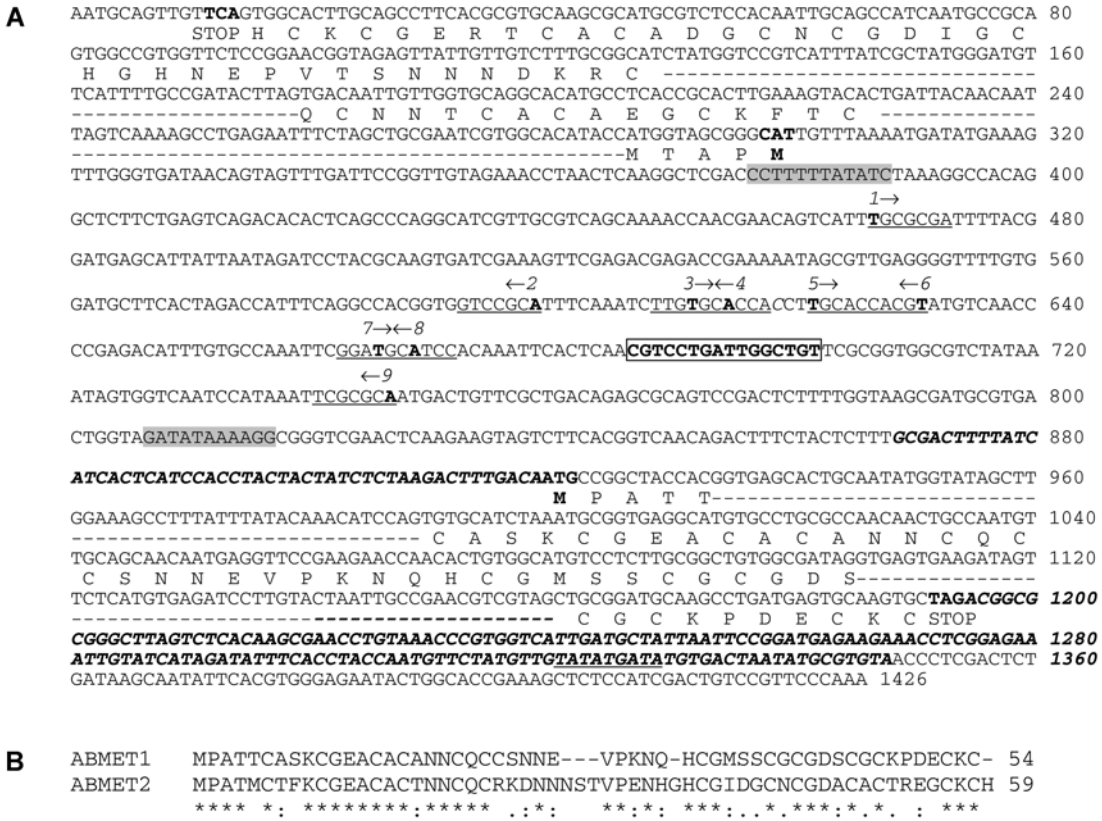

Fig. 1. Genomic DNA sequence and amino acid sequence comparison of metallothioneins 1 and 2.
A. Chromosomal genomic DNA sequence of *met*1 (nucleotides 920 to 1196) and *met*2 (nucleotides 300 to 12) genes from *Agaricus bisporus* C54-carb8 genomic Lawrist cosmid library DNA. Deduced amino acid sequence is shown below the corresponding nucleotide sequence, start and stop codons are indicated in bold, and introns are identified by dashed lines in the deduced amino acid sequence. Bold italicized nucleotides indicate *met*1 5' and 3' untranslated cDNA sequences, and the putative polyadenylation signal sequence is underlined. Putative TATA motif is identified by a shaded box, metal responsive elements are numbered and underlined, the starting nucleotide of each is indicated in bold, and the arrow indicates the direction of each numbered motif. Conserved CAAT motif is in bold and boxed. **B**. Alignment of *Agaricus bisporus* (AB) MET1 and MET2 amino acid sequences. The asterisk identifies identical bases, double dots indicate conserved substitutions, and single dots indicate semiconserved substitutions.

possible from the *P. placenta* genomic sequence to discern further conserved features, such as introns and start and stop codons, which would allow description of an *mt*-like gene. In each case, it appeared the *P. placenta* sequences belonged to a larger peptide unrelated to metallothionein. For *P. chrysosporium*, neither mt-like protein had been previously identified as a putative open reading frame. In *L. bicolor*, one sequence had been identified (*L. bicolor* database, Accession No. 314477); this was despite both *L. bicolor* sequences being very similar at the nucleotide level. Only in *C. cinerea* had two *mt*-like sequences been identified as predicted proteins (Accession No. A8PBG0 and A8N9Z8). None of the predicted proteins that were identified had been ascribed a putative metallothionein function.

Unlike *A. bisporus*, the *met* genes in the other basidiomycetes were not organized as bidirectional gene pairs and were located on different contigs in the genome assemblies. The amino acid sequences of the *met* genes identified were aligned with sequences previously described from *G. margarita* and *Paxillus involutus* [2] (Fig. 2A). Analysis showed conservation of cysteine residues, particularly in the C-terminal peptide domain common to these fungi.

Analysis of the putative promoter regions of the *C. cinerea*, *L. bicolor*, and *P. chrysosporium* putative *met* genes identified numerous metal response element motifs (MRE). In the region 1,000 bp downstream from the putative translational start codon, the two *C. cinerea* putative *met* genes had 13 and 19 MREs, *L. bicolor* 12 and 14, and *P. chrysosporium* 15 and 16 (data not shown). A further conserved motif was identified centered on a putative CCAAT-box motif (Fig. 2B). The motif was present 214 bp downstream from the *A. bisporus met*1 translational start codon and in both putative *met* sequences from *C. cinerea*. A conserved CCAAT region was also identified in *L. bicolor*, but there was little similarity with *A. bisporus* or *C. cinerea* outside the core consensus sequence. No corresponding motif was identified in *P. chrysosporium*. Interestingly, the motif in both putative *C. cinerea met* promoters was flanked by MRE motifs, and in *A. bisporus* both MRE motifs differed from the consensus sequence by one base change.

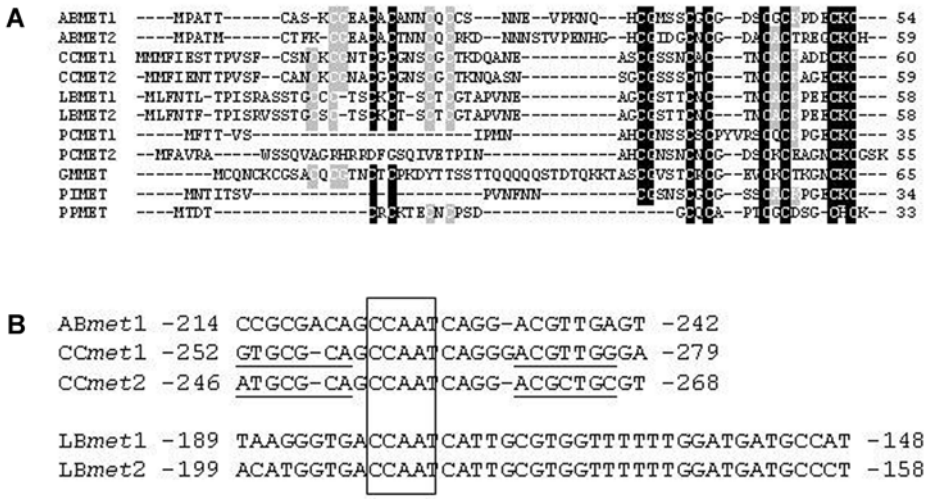

Fig. 2. Comparison of mt-like sequences and promoter region alignment.
A. Alignment of *Agaricus bisporus* (AB) MET1 and MET2 sequence with *mt*-like sequences from *Coprinopsis cinerea* (CC, Accession No. CCMET1: A8PBG0, and CCMET2: A8N9Z8), *Lacaria bicolor* (LB, *L. bicolor* database Accession No. 314477), *Phanerochaete chrysosporium* (PC), *Gigaspora margarita* (GM, Accession No. Q8NJ33), *Paxillus involutus* (PI, Accession No. AAS19463), and *Postia placenta* (PP). The black box indicates residues conserved in a least seven sequences, gray shading amino acids are conserved in 5 or 6 sequences. **B**. Alignment of conserved CCAAT-box motif from *Agaricus bisporus* (AB), *Coprinopsis cinerea* (CC), and *Lacaria bicolor* (LB) *mt*-like gene promoter sequences. The position downstream from the translational start site is given for each sequence. The CCAAT motif is boxed and metal responsive elements (MRE) are underlined.

Metallothionein Gene Expression During Fruiting Body Post-harvest Storage and Initiation

The *A. bisporus met*1 gene had been previously reported to be up-regulated following harvest and 2 day storage of fruiting bodies [12]. Gene expression experiments were conducted to determine the change in transcript levels over a wider range of time points and between stipe, cap, and gill tissues during post-harvest storage. The aim was to determine the point at which increased transcription was initiated, the regulation of gene expression during longer term storage, and whether any spatial variation in expression exists.

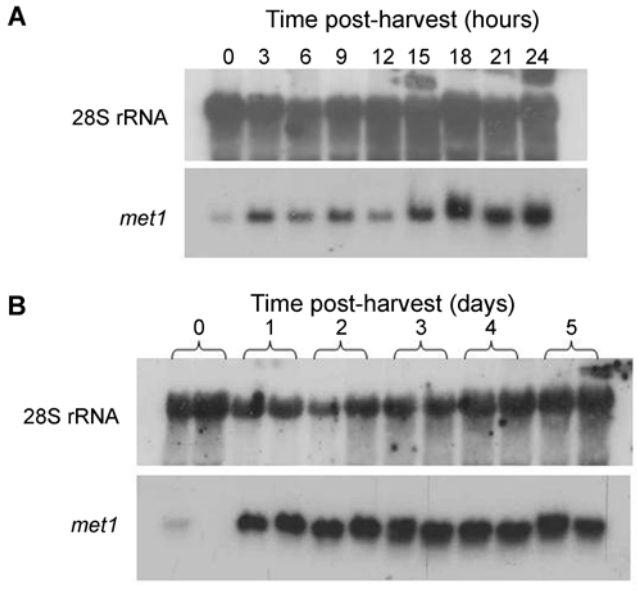

Fig. 3. Northern analysis of *Agaricus bisporus met*1 transcripts in fruiting bodies during post-harvest storage (18°C, 95% relative humidity).
A. For the first 24 h following harvest, sampled at 3 hourly intervals. **B**. Over 5 days storage, sampled at 24 h intervals. The upper Northern image for each treatment corresponds to the 28S rRNA loading control for each sample.

The data revealed a low level of *met*1 transcription at the point of harvest (time=0 h), while increased transcription was detected within 3 h of harvest and the trend of increasing transcription continued during the first 24 h of storage (Fig. 3A). The transcript levels of *met*1 then stabilized to a relatively constant level for the subsequent 5 days of post-harvest storage (Fig. 3B).

Analysis of *met*1 transcription between the different tissues of the mushrooms during post-harvest storage showed a similar pattern of expression between the stipe and cap tissues (Fig. 4A). In each case, transcription increased between time 0 and 24 h storage and remained constant after 48 h. Transcription in gill tissue was very low and a slight signal was detected at 48 h following prolonged exposure with the autoradiography film (Fig. 4A).

Northern analysis was also performed using a *met*2 gene-specific probe, but no transcripts were detected (data not shown). The increased sensitivity of qRT–PCR was used to determine whether *met*2 transcription could be detected during post-harvest storage and fruiting body initiation and compared with *met*1. Stipe, cap, and gill tissue samples were tested for transcription at harvest and after 2 days post-harvest storage. The data showed that the pattern of *met*2 transcription mirrored that of *met*1 (increasing during

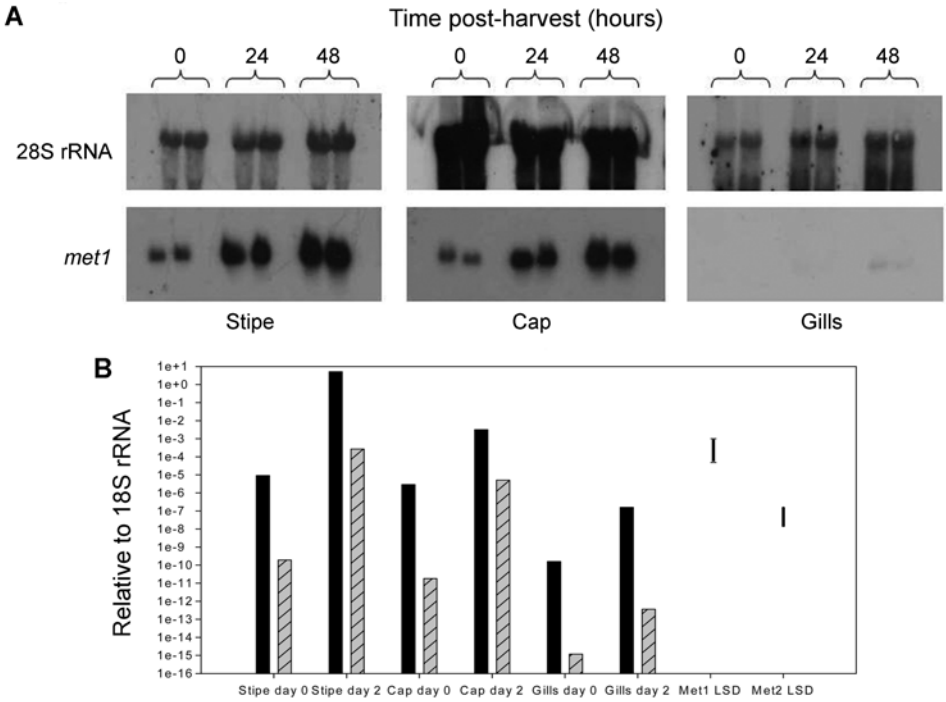

Fig. 4. Qualitative and quantitative analyses of the metallothionein transcripts.
A. Northern analysis of *Agaricus bisporus met*1 transcripts in fruiting bodies sampled at harvest (0 h) and after 24 and 48 h storage (18°C, 95% relative humidity), and dissected into stipe, cap, and gill tissue. The upper Northern image for each treatment corresponds to the 28S rRNA loading control for each sample. **B**. Mean quantitative RT–PCR ($\log_{10}$-transformed) analysis of *Agaricus bisporus met*1 and *met*2 transcripts in stipe, cap, and gill tissue at harvest and following 2 days storage (18°C, 95% relative humidity). Vertical lines show LSD (5%, 10 d.f.) for both *met*1 and *met*2 for comparing means at different times, as calculated from the residual mean square obtained from the ANOVA.

storage), where the levels were significantly lower for *met*2 (at least two orders of magnitude lower) (Fig. 4B). The qRT–PCR of samples taken during fruiting body initiation showed that *met*1 and *met*2 had a similar pattern of transcription (*i.e*., increasing expression as fruiting bodies are formed), but the level of *met*2 was at least four orders of magnitude lower (data not shown) and transcription of both *met*1 and *met*2 was significantly lower than that observed during post-harvest storage (data not shown).

qRT–PCR control experiments showed no evidence of active reverse transcription or contaminating DNA in the reactions (data not shown). Specificity of the primers was confirmed by melting curve analysis, which showed that a single product was obtained and standard curves demonstrated that the PCR was operating at close to 100% efficiency (data not shown).

Discussion

This paper further characterizes the *Agaricus bisporus met*1 gene shown previously to be upregulated in mushrooms following post-harvest storage [12]. Genomic DNA sequence information was presented and a second small cysteine-rich metallothionein-like (*mt*-like) gene (*met*2) was identified downstream from *met*1 with a bidirectional gene pair organization [44]. This is in contrast with the tandemly repeated metallothionein gene organization described in yeast systems [28]. Both *met*1 and *met*2 showed sequence similarity (57.8% amino acid) and TATA box and intron conservation, suggesting that the gene pair arose as a consequence of a gene duplication event. Interestingly, *A. bisporus* was the only fungus studied where the *mt*-like sequences shared a promoter and this might have indicated a recent duplication event, as the genes had not been separated by recombination. However, two similar *mt*-like sequences were identified in other basidiomycete fungi that did not share a common promoter and, therefore, suggests the duplication occurred earlier in the basidiomycete lineage. The position of the first intron of *met*1 and *met*2 in relation to the translational start codon was conserved (15 bp from the start ATG), despite showing little sequence similarity and a difference in length. It is not clear whether this conservation is necessary for gene function or if it is an artifact of gene duplication. Intron conservation has been described in the *A. bisporus* argininosuccinate lyase gene [48], and the presence of introns has been identified as important in obtaining transgene RNA stability and protein activity in homobasidiomycetes [4, 27, 40]. Further study would be required to determine whether the positioning of the intron is relevant to *met* gene function in *Agaricus bisporus*.

*A. bisporus met*1 and *met*2 sequences were different from the metallothionein gene sequence previously described in *A. bisporus* (Accession No. P04358) [31]. Both genes showed a cysteine conservation pattern similar to metallothionein-like proteins identified in *Gigaspora margarita* (*Gmar*MT1, Accession No. Q8NJ33) [24], *Paxillus involutus* (PiMT1, Accession No. AAS19463) [2], and *Hebeloma cylindrosporum* (Hc*MT1* and Hc*MT2*, accession numbers EU0049884 and EU049885, respectively) [35]. The *G. margarita* and *P. involutus* proteins functionally complimented yeast mutants Δ*yap*1 and Δ*cup*1/2, which cause cadmium and copper sensitivity, respectively. In *H. cylindrosporum*, the two metallothionein-like proteins were shown to function differently; Hc*MT1* increased copper resistance and Hc*MT2* resulted in both copper and cadmium resistance in the yeast strains tested [35]. Lanfranco *et al.* [24] described the *Gmar*MT1 protein as a novel class of fungal metallothionein-like polypeptides, which could not be categorized into any described subfamily, but showed some similarity with plant metallothioneins. Their description of a class of fungal MT-like polypeptides identified by repeated C-X-C motifs is supported by the identification of similar sequences in the genome sequence of *Coprinopsis cinerea*, *Laccaria bicolor*, and *Phanerochaete chrysosporium*.

Transcript analysis clearly demonstrated the rapid upregulation of *met*1 in *A. bisporus* fruiting bodies within 3 hours from harvest (Fig. 3A), indicating an early or primary response to harvesting, and is in contrast with later transcriptional events described previously [13]. The level of transcripts was subsequently maintained during storage (Fig. 3B). Studies of harvested and cold-stored apples identified the upregulation of two *mt*-like genes within 45 min of harvest [37]. It is likely that primary response genes are initially transcribed as a consequence of the stress of harvesting, but subsequent transcript levels are influenced by secondary stimuli such as available nutrient levels.

The promoter of the *A. bisporus met* genes contained eight putative MRE motifs, which suggests that their transcription would be influenced by the level of metal ions in the cell. In the post-harvest mushroom, increased levels of metal ions in the cytosol might result from the breakdown of metal-containing proteins. Protein levels have been shown to fall by over 90% in mushrooms following harvest [18], and have been linked to the increased activity of the protein serine proteinase [5, 23]. Since many enzymes employ metal cofactors, the rapid turnover of these proteins post-harvest would cause a large influx of metal ions into the cytosol. We suggest, therefore, that *met*1 is upregulated to prevent the cellular damage such metal ions would cause to the cell. This hypothesis is supported further by the differential expression of *met*1 between the different tissues of the mushroom (Fig. 4), where significantly lower transcripts were observed in the gills compared with the cap and stipe. The gill tissues are physiologically distinct from the stipe and cap, and may be described as a nutrient "sink." Nutrients are transported from the stipe and cap tissues, in which protein turnover and serine proteinase activity are greater [5], to allow the gills to carry out their function of spore production unperturbed. As a consequence, it would be unlikely for there to be a build-up of harmful metal ions in the cytosol of gill cells and would account for the low expression of *met*1 in these tissues.

Bidirectional gene pairs are commonly considered as a mechanism by which genes with related function may be coregulated, resulting in the conservation of the divergent gene organization [22, 44, 46]. Plant and animal genomes contain many such gene organizations and are thought to be an important adaptation to regulating gene expression [21, 34, 36]. Northern analysis of *met*2 transcription did not produce a detectable signal. Therefore, qPCR was employed because of the greater sensitivity of the method. The data show the expression pattern of *met*2 had the same tissue distribution as *met*1 in the post-harvest mushroom, but the level of transcription was greatly reduced (Fig. 4B), suggesting that the promoter is organized to preferentially generate *met*1 transcripts. The reason for this directionality is not understood and there is no evidence from *in silico* analysis to conclude why MET1 might be functionally different from MET2. Metallothionein genes in *H. cylindrosporum* were shown to exhibit differing patterns of mRNA induction and expression, but it is not known whether these genes are bidirectionally arranged [35]. Analysis of the promoters of bidirectional human genes has shown that regulation can be achieved with a single transcription factor binding site [25, 44]. Furthermore, human bidirectional genes were shown to have the CCAAT-box motif overrepresented in their promoters, although this may have been linked to an underrepresentation of a TATA-box [25]. The CCAAT-box was symmetrically placed in bidirectional promoters and in a position conserved from the transcriptional start site [25]. A conserved CCAAT-box was identified in the *A. bisporus met* promoter downstream of the *met*1 transcriptional start site, but was absent for *met*2. This may explain the directionality of the promoter, although confirmation would require further study.

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